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Hua, H ; Georgiev, O ; Schaffner, W ; Steiger, D

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Human copper transporter Ctr1 is functional in *Drosophila*, revealing a high degree of conservation between mammals and insects

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Abstract Living cells have to carefully control the intracellular concentration of trace metals, especially of copper, which is at the same time essential but owing to its redox activity can also facilitate generation of reactive oxygen species. Mammals have two related copper transporters, Ctr1 and Ctr2, with Ctr1 playing the major role. The fruit fly *Drosophila* has three family members, termed Ctr1A, Ctr1B, and Ctr1C. Ctr1A is expressed throughout development, and a null mutation causes lethality at an early stage. Ctr1B ensures efficient copper uptake in the intestinal tract, whereas Ctr1C is mainly expressed in male gonads. Ectopic expression of Ctr1 transporters in *Drosophila* causes toxic effects due to excessive copper uptake. Here, we compare the effects of human Ctr1 (hCtr1) with those of the *Drosophila* homologs Ctr1A and Ctr1B in two overexpression assays. Whereas the overexpression of *Drosophila* Ctr1A and Ctr1B results in strong phenotypes, expression of hCtr1 causes only a very mild phenotype, indicating a low copper-import efficiency in the *Drosophila* system. However, this can be boosted by coexpressing the human copper chaperone CCS. Surprisingly, hCtr1 complements a lethal Ctr1A mutation at least as well as

Ctr1A and Ctr1B transgenes. These findings reveal a high level of conservation between the mammalian and insect Ctr1-type copper importers, and they also demonstrate that the *Drosophila* Ctr1 proteins are functionally interchangeable.

Keywords Ctr1 · Copper · Copper import · *Drosophila*

Introduction

Copper is essential for normal cell functionality in all eukaryotic organisms owing to its capacity to shift between two transition states, Cu(I) and Cu(II). It is present within redox enzymes such as Cu/Zn superoxide dismutase, tyrosinase, and cytochrome *c* oxidase. Optimal copper binding is crucial for the activity of these enzymes. On the other hand, when free copper accumulates in the cell, it contributes directly to the production of reactive oxygen species via the Fenton reaction, resulting in oxidative damage to DNA, proteins, and lipids [1, 2]. Eukaryotic organisms from yeast to humans use elaborate systems to regulate copper homeostasis [2–5]. In eukaryotes, copper is imported as Cu(I) by high-affinity copper transporters of the Ctr family. These presumably form homotrimeric complexes in the membrane and acquire copper in an ATP-independent manner [6]. In yeast, three Ctr family members were characterized: yCtr1, yCtr2, and yCtr3. Studies in yeast revealed that yCtr1 and yCtr3 localize to the plasma membrane and import extracellular copper into the cytoplasm [7, 8]. Excess copper is stored in the vacuole, from where it is imported back into the cytoplasm upon copper starvation by yCtr2 [9]. Humans and mice have two Ctr proteins, Ctr1 and Ctr2. Mouse Ctr1 has been shown to be essential for copper uptake and delivery. Ctr2, which

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localizes to endosomes/lysosomes, also contributes to copper uptake [10–13]. *Drosophila* contains three Ctr family members, designated Ctr1A, Ctr1B, and Ctr1C [14] (see Fig. S1 for a protein sequence alignment of the three proteins). Ctr1A is constitutively expressed from 4-h-old embryos to adults. A Ctr1A null mutation causes embryonic lethality. Ctr1B is mainly expressed in the intestine. The Ctr1B mutant flies are viable but highly sensitive to copper depletion and, to a lesser extent, to copper load [14, 15]. Of note, the Ctr1B gene is transcriptionally upregulated upon copper starvation [15]. Besides the Ctr1 importers, copper can also be acquired via less specific, low-affinity metal transporters, such as divalent metal transporter 1 (DMT1) [16–18]. Although the subcellular localization and expression patterns of these copper transporters have been studied in detail, the functional divergence among Ctr1 family members has not been intensively examined. Here, we report that human Ctr1 (hCtr1), which shows very weak copper-import activity when expressed in *Drosophila*, can complement a null mutation of *Drosophila* Ctr1A, revealing a high degree of conservation between mammalian and insect Ctr1 proteins. We furthermore show that also Ctr1B can rescue the Ctr1A mutants, provided that expression levels are not too high. The finding that Ctr1B can functionally replace Ctr1A implies that the differences between Ctr1A and Ctr1B are based on the regulation of their expression, but not on the molecular function of the respective proteins.

Materials and methods

Fly culture

One liter of standard fly food was composed of 55 g corn, 10 g wheat, 100 g yeast, 75 g glucose, 8 g agar, and 15 ml antifungal agent nipagin (15% in ethanol). For experiments, food was supplemented with CuSO₄ or bathocuproine disulfonate (BCS) disodium salt hydrate (Sigma-Aldrich no. 14,662-5) at the concentrations indicated. BCS is a specific copper chelator used to deplete copper in the food. Flies were raised at 25 °C and 65% humidity.

Plasmids and transgenic fly strains

To introduce the transgenes into the same locus, the phage C31 site-specific integration system was used. A pUAST-AttB-plus vector was made by adding an AttB site into the P element transformation vector pUAST [19]. The coding sequences of human *Ctr1* (GenBank CCDS6789.1) and human *CCS* (GenBank CCDS8146.1) were cloned into the pUAST-AttB-plus vector. The plasmids were injected into flies harboring an AttP site (AttP 51D), resulting in

UAS-hCtr1[AttP 51D] or *UAS-hCCS*[AttP 51D] flies, respectively. The coding sequence of *Ctr1A* (GenBank NM_132108) was cloned into the previously described vector pUASTattB (GenBank EF362409.1). The construct was introduced into two different AttP sites (AttP 51D and AttP 86Fb), resulting in the transgenic lines *UAS-Ctr1A*[AttP 51D] and *UAS-Ctr1A*[AttP 86Fb]. The *UAS-Ctr1B* (AS24e) strain has been described previously [15]. For the *UAS-Ctr1B* (B6) and *UAS-Ctr1B* (B12) strains, the Ctr1B coding sequence used in the AS24e strain was subcloned into a pUASP vector and transgenic flies were produced using standard P element transgenesis [20].

Ubiquitous expression of Ctr1 copper transporters

Ubiquitous expression of hCtr1, Ctr1A, and Ctr1B was achieved by crossing *UAS-hCtr1*[AttP 51D], *UAS-Ctr1A*[AttP 51D], and *UAS-Ctr1B* (AS24e) flies with flies having a strong and constitutive actin-GAL4 driver. The crosses were made with the food conditions indicated and the number of adult flies undergoing eclosion was counted. Survival was calculated relative to the numbers of siblings (reference genotype flies) undergoing eclosion which carried a balancer chromosome instead of the actin-GAL4 driver transgene (CyO, y⁺ in Fig. 1a, *TM3* in Fig. 1b) but otherwise had identical genotypes.

The genotypes were as follows (with the total number of reference genotype flies in parentheses). Figure 1a, normal food: Ctr1A (AttP 51D), y w; *UAS-Ctr1A*[AttP 51D]/*actin-GAL4*; + (228); Ctr1B (AS24e), y w; *UAS-Ctr1B*[AS24e]/*actin-GAL4*; + (171); hCtr1 (AttP 51D), y w; *UAS-hCtr1*[AttP 51D]/*actin-GAL4*; + (144). Figure 1a, BCS: Ctr1A (AttP 51D), y w; *UAS-Ctr1A*[AttP 51D]/*actin-GAL4*; + (222); Ctr1B (AS24e), y w; *UAS-Ctr1B*[AS24e]/*actin-GAL4*; + (98); hCtr1 (AttP 51D), y w; *UAS-hCtr1*[AttP 51D]/*actin-GAL4*; + (147). Figure 1b, normal food: hCtr1 (AttP 51D), y w; *UAS-hCtr1*[AttP 51D]/+; *actin-GAL4*/+ (153). Figure 1b, copper: hCtr1 (AttP 51D), y w; *UAS-hCtr1*[AttP 51D]/+; *actin-GAL4*/+ (140).

Eye-specific expression assay

To direct the eye-specific expression of hCtr1, human copper chaperone CCS (hCCS), Ctr1A, and Ctr1B, flies containing the respective transgenes were crossed with an eye-specific GMR-GAL4 driver and raised on the media indicated. Adult animals were collected and eye pictures were acquired with a LEICA MZ16 microscope and a LEICA DFC280 camera.

The genotypes were as follows. Figure 2a: Ctr1A (AttP 51D), y w; *UAS-Ctr1A*[AttP 51D]/*GMR-GAL4*; +; Ctr1A (AttP 86Fb), y w; *GMR-GAL4*/+; *UAS-Ctr1A*[AttP 86Fb]/+; Ctr1B (AS24e), y w; *UAS-Ctr1B*[AS24e]/*GMR-GAL4*;

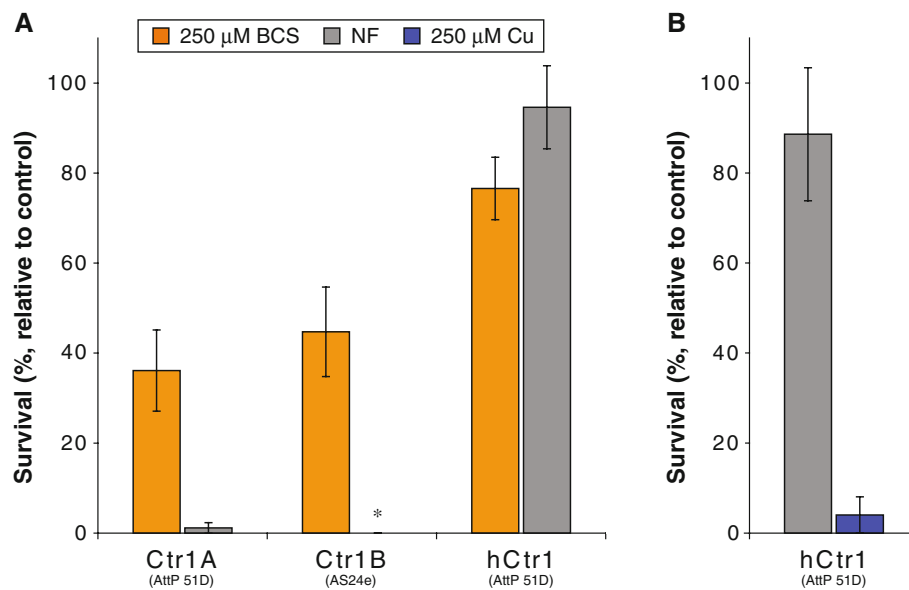


Fig. 1 Ubiquitous expression of Ctr copper transporters in transgenic *Drosophila*. The transgenes indicated were expressed under the control of *UAS* enhancers, by the strong and ubiquitous *GAL4* driver actin-*GAL4*. Flies were raised on normal food (*NF*) and food containing 250 μM bathocuproine disulfonate (*BCS*) (a) or on *NF* and food containing 250 μM CuSO_4 (b) and the number of adult flies undergoing eclosion was counted. Although overexpression of *Ctr1A* and *Ctr1B* led to strong lethality on *NF* which could be partially

rescued by raising the flies on 250 μM *BCS*, overexpression of the gene for human *Ctr1* (*hCtr1*) did not decrease viability on *NF* (a). At a high concentration of copper (250 μM), overexpression of *hCtr1* also strongly reduced survival (b). Average survival rates for three experiments are given [two experiments for *Ctr1B* (*BCS*), *hCtr1* (*BCS*) in a and for *hCtr1* (*NF*) in b]. Error bars indicate standard errors. Data points with zero value are indicated with an asterisk

+; *hCtr1* (AttP 51D), *y w*; *UAS-hCtr1*[AttP 51D]/*GMR-GAL4*; +. Figure 2b: *hCCS*, *y w*; *UAS-hCCS*[AttP 51D]/+; *GMR-GAL4*/+; *hCtr1*, *y w*; *UAS-hCtr1*[AttP 51D]/+; *GMR-GAL4*/+; *hCtr1 hCCS*, *y w*; *UAS-hCtr1*[AttP 51D]/+; *UAS-hCCS*[AttP 51D]; *GMR-GAL4*/+.

Rescue assay

For the rescue experiment, a null mutant allele of *Ctr1A* (*Ctr1A*²⁵) was used [21]. *hCtr1*, *Ctr1A*, and *Ctr1B* transgenes were ubiquitously expressed using a moderately strong *tub-GAL4* driver in hemizygous *Ctr1A*²⁵/*Y* males and the number of rescued animals was scored. The survival rate was calculated as described earlier, namely, the ratio between flies with the indicated genotype and flies with the reference genotype was calculated. The reference flies were *Ctr1A*²⁵/*FM7*; *UAS-transgene*/+; *MKRS*/+ females originating from the same cross (these females carry the X chromosome balancer *FM7* and the third chromosome balancer *MKRS* and show wild-type survival).

The genotypes were as follows (with the total number of reference females given in parentheses). *Ctr1A* (AttP 51D), *w Ctr1A*²⁵/*Y*; *UAS-Ctr1A*[AttP 51D]/+; *tub-GAL4*/+ and *w Ctr1A*²⁵/*Y*; *UAS-Ctr1A*[AttP 51D]/+; *MKRS*/+ (71). *Ctr1A* (AttP 86Fb), *w Ctr1A*²⁵/*Y*; +; *tub-GAL4*/+; *UAS-Ctr1A*[AttP 86Fb] and *w Ctr1A*²⁵/*Y*; +; *MKRS*/+ (76).

hCtr1 (AttP 51D), *w Ctr1A*²⁵/*Y*; *UAS-hCtr1*[AttP 51D]/+; *tub-GAL4*/+ and *w Ctr1A*²⁵/*Y*; *UAS-hCtr1*[AttP 51D]/+; *MKRS*/+ (56). *Ctr1B* (AS24e), *w Ctr1A*²⁵/*Y*; *UAS-Ctr1B*[AS24e]/+; *tub-GAL4*/+ and *w Ctr1A*²⁵/*Y*; *UAS-Ctr1B*[AS24e]/+; *MKRS*/+ (49). *Ctr1B* (B6), *w Ctr1A*²⁵/*Y*; +; *UAS-Ctr1B*[B6]/*tub-GAL4* and *w Ctr1A*²⁵/*Y*; +; *UAS-Ctr1B*[B6]/*MKRS* (25). *Ctr1B* (B12), *w Ctr1A*²⁵/*Y*; *UAS-Ctr1B*[B12]/+; *tub-GAL4*/+ and *w Ctr1A*²⁵/*Y*; *UAS-Ctr1B*[B12]/+; *MKRS*/+ (12).

Results

hCtr1 shows weak copper toxicity in *Drosophila*

We examined if *hCtr1* could function as a copper importer in an ectopic insect environment in two assays that had been previously used to characterize the *Drosophila* intestinal copper importer *Ctr1B*. Ubiquitous and constitutive overexpression of a *UAS-Ctr1B* complementary DNA transgene (the P element insertion line AS24e) by actin-*GAL4* led to complete larval and pupal lethality when the flies were raised on normal food. The observed lethality is copper-dependent, since a large fraction of the flies successfully reached the adult stage if they were raised on food containing the copper-specific metal chelator *BCS*,

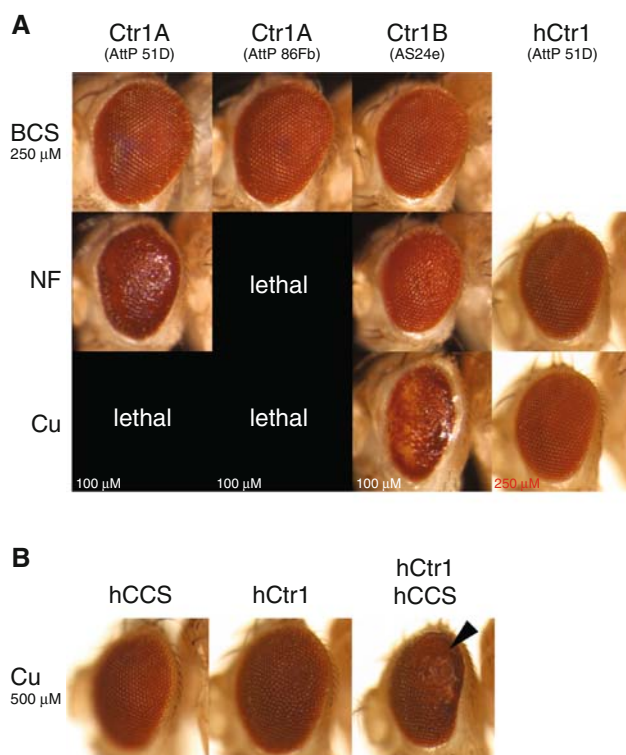


Fig. 2 Eye-specific expression of Ctr copper transporters in transgenic *Drosophila*. Expression of *Drosophila* Ctr1 transporters, but not of hCtr1, elicits strong copper-dependent phenotypes (a). Overexpression of hCtr1 only caused a rough eye phenotype when human copper chaperone CCS (*hCCS*) was coexpressed (b). Flies which expressed the transgenes indicated under control of the eye-specific GMR-GAL4 driver were raised on either NF or on food supplemented with BCS or copper. Adults were collected and eye pictures were acquired (lens magnification $\times 5$, for hCtr1 pictures $\times 10$). **a** Overexpression of Ctr1A and Ctr1B transgenes for flies raised on NF causes rough eye phenotypes and lethality, both of which could be rescued with 250 μ M BCS. Overexpression of *Ctr1A*[*AttP 51D*] in flies raised on NF led to almost complete lethality; one of the few “escapers” is shown. Overexpression of *Ctr1A*[*AttP 86Fb*] in flies raised on NF and of both *Ctr1A* transgenes in flies raised on 100 μ M copper caused complete lethality. In contrast, hCtr1-expressing flies had wild-type eyes and survived well both on NF and on 250 μ M copper. **b** Coexpression of the hCCS strongly enhances the effect of hCtr1 (the arrow indicates the severe distortion in the dorsal eye area). Overexpression of hCtr1 alone caused a slight rough eye phenotype in flies raised on 500 μ M copper. hCCS did not cause a mutant eye phenotype on its own

i.e., on food deprived of bioavailable copper [15]. In a second assay, Ctr1B was expressed under the control of the eye-specific *GMR* promoter. This caused a distorted eye development, manifested as a “rough eye” phenotype which could be enhanced by raising the flies on copper-supplemented food and could be reversed either by raising them on BCS-supplemented food or by coexpression of the copper exporter DmATP7. This demonstrates that the observed rough eye phenotype is due to excess copper import [22].

To compare hCtr1 with its closest *Drosophila* homolog, Ctr1A [21], we cloned the complementary DNAs of these genes into the *UAS-transgene-SV40polyA* expression cassette previously used for Ctr1B studies and inserted the cassette, using the phiC31 transgenesis system, into defined AttP landing sites [23].

In the actin-GAL4 overexpression assay, Ctr1A behaved almost like Ctr1B: on normal food, overexpression resulted in almost complete lethality and survival could be restored on BCS-supplemented food (Fig. 1a). In contrast, hCtr1-expressing flies survived perfectly well on normal food. Only with high concentrations of copper (250 μ M) did hCtr1 expression reduce the survival rate (Fig. 1b). Note that the difference in the phenotype strength between hCtr1 and Ctr1A is unlikely to be attributed to differences in transcription rates which occur with traditional P element transgenes due to position effects, since both transgenes share the same expression cassette and are inserted into the same chromosomal site (AttP 51D).

We also compared the effects of hCtr1, Ctr1A, and Ctr1B in the GMR-GAL4 eye-specific overexpression assay (Fig. 2a). Ctr1B overexpression caused a rough eye phenotype in flies raised on normal food. This phenotype was strongly aggravated in flies raised on 100 μ M copper. Interestingly, the strong rough eye phenotype was accompanied by high lethality. This effect is most likely due to expression of the GMR-GAL4 driver in tissues essential for survival. Alternatively, it might be due to a nonautonomous effect of the eye-specific overexpression. Expression of Ctr1A led to very severe rough eye phenotypes and almost complete lethality for flies raised on normal food. With overexpression of both Ctr1A and Ctr1B, a complete restoration of wild-type eye phenotype and wild-type survival could be observed for flies raised on 250 μ M BCS, indicating that the effects described are copper-dependent. With hCtr1, a mild rough eye phenotype could be induced only when 500 μ M copper was used (Fig. 2b).

Taken together, these data demonstrate that hCtr1 can function as a copper importer in the *Drosophila* system, albeit with a considerably lower efficiency compared with the *Drosophila* importers Ctr1A and Ctr1B.

hCtr1 copper-import function can be enhanced by coexpression of hCCS

The apparently weak copper-import function of hCtr1 in *Drosophila* might be explained by evolutionary divergence of the partner proteins needed for copper trafficking, e.g., leading to improper delivery of copper to the *Drosophila* copper chaperones. Consistent with this notion of evolutionary divergence, we found that coexpression of the

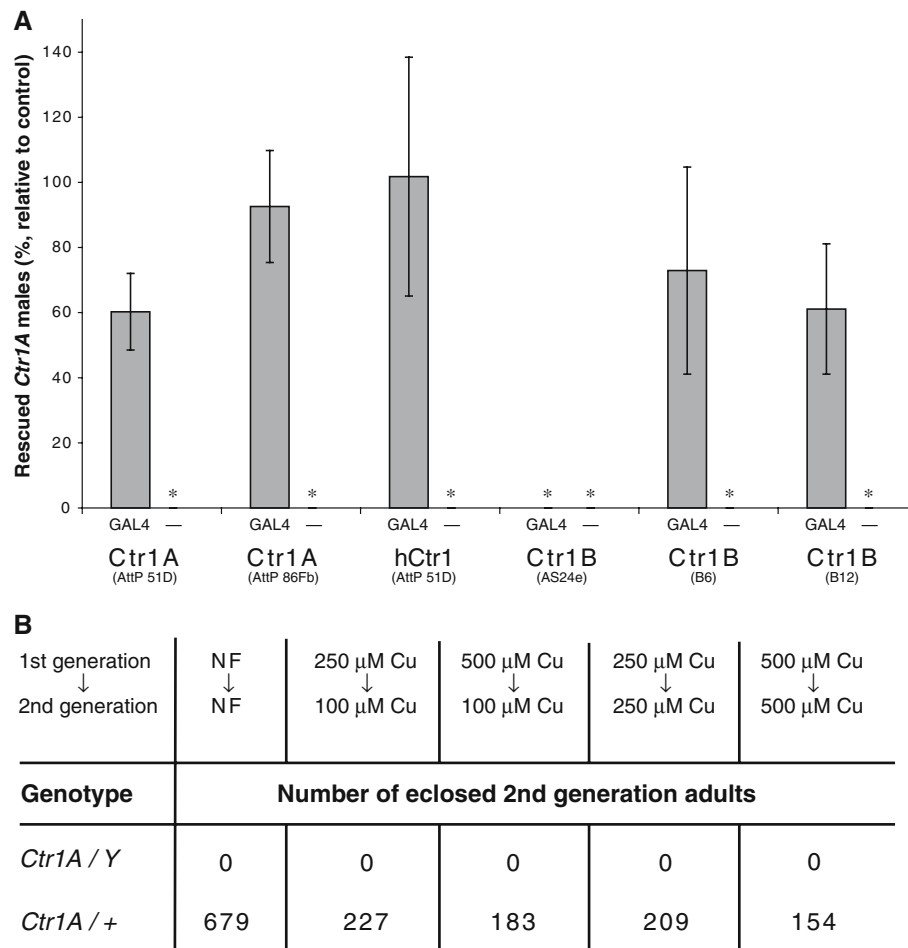


Fig. 3 hCtr1 fully complements a *Drosophila* Ctr1A null mutant. hCtr1, Ctr1A, and Ctr1B transgenes were ubiquitously expressed with the moderately strong tub-GAL4 driver in hemizygous *Ctr1A*²⁵/Y males and the number of adult (rescued) animals was scored (**a**). Bars labeled with GAL4 indicate the fraction of *Ctr1A*²⁵/Y; *UAS-transgene*/+; *tub-GAL4*/+ males relative to *Ctr1A*²⁵/FM7; *UAS-transgene*/+; *MKRS*/+ females originating from the same cross and showing wild-type survival. Bars labeled with a hyphen indicate the fraction of *Ctr1A*²⁵/Y; *UAS-transgene*/+; *MKRS*/+ males (lacking the GAL4 driver and therefore not expressing the *UAS-transgene*) relative to *Ctr1A*²⁵/FM7; *UAS-transgene*/+; *MKRS*/+ females. Each bar shows the average relative survival from at least three independent experiments. Error bars indicate standard errors. Data points for

zero survival are marked with an asterisk. Although Ctr1 transgenes were able to complement, copper supplementation in the food could not rescue the *Ctr1A*²⁵ mutants, even if supplementation was performed over two generations (**b**). For the first generation, *Ctr1A*²⁵/FM6 females were crossed to FM6/Y males with the food conditions indicated. Among the offspring of these crosses, no *Ctr1A*²⁵/Y sons were present. For the second generation, *Ctr1A*²⁵/FM6 daughters and FM6/Y sons were crossed with the food conditions indicated. The number of *Ctr1A*²⁵/Y males and the number of *Ctr1A*²⁵/FM6 females (heterozygous for *Ctr1A*²⁵) undergoing eclosion from these second crosses are listed. With a full rescue, the ratio between these genotypes would be 1:1, according to Mendelian expectations

hCCS with hCtr1 resulted in a stronger rough eye phenotype in the GMR-GAL4 assay (Fig. 2b). This cooperation between human copper importer and human copper chaperone is synergistic, since expression of hCCS alone did not result in a mutant eye phenotype.

hCtr1 complements the lethal *Drosophila* *Ctr1A*²⁵ mutation

In the overexpression assays described earlier, the *Drosophila* importers Ctr1A and Ctr1B showed similar strong, copper-dependent phenotypes. In comparison, the effect of

hCtr1 was very mild. We therefore wondered if a hCtr1 transgene would be able to complement a loss of Ctr1A. In this complementation assay, we used a null mutant of the Ctr1A gene (*Ctr1A*²⁵). Animals lacking Ctr1A arrest in early larval stages and are deficient in the activity of copper-dependent enzymes [21]. We asked whether overexpression of the Ctr1 transgenes in a *Ctr1A*²⁵ mutant background could overcome the developmental arrest (Fig. 3a). To avoid the lethality of Ctr1A and Ctr1B overexpression caused by the strong GAL4 driver actin-GAL4, we employed the tubulin-GAL4 driver, which has a more moderate but still ubiquitous and constitutive

expression. In this setup, overexpression of Ctr1A could rescue the *Ctr1A*²⁵ mutant phenotype, allowing the flies to normally reach the adult stage. Strikingly, hCtr1 rescued at least as well, whereas the Ctr1B line AS24e which was used in the overexpression assays (see above) was unable to rescue the *Ctr1A*²⁵ mutant phenotype. We also tested two further Ctr1B P element transgenes, the lines B6 and B12, which show milder phenotypes compared with AS24e in the actin-GAL4 and GMR-GAL4 assays (data not shown). With these transgenes, the *Ctr1A*²⁵ mutants could be rescued. Similar results were obtained with Ctr1C P element transgenes: insertions which exhibit strong toxicity showed no rescue, whereas “milder” lines could rescue (data not shown). It is likely that the lethality seen with “strong” Ctr1B and Ctr1C transgenes is not due to a failure to rescue copper uptake in *Ctr1A*²⁵ mutants, but is rather due to toxicity caused by excessive copper uptake. Consistent with such an explanation, *Ctr1A*²⁵ mutants rescued by Ctr1A or by the Ctr1B and Ctr1C lines were consistently smaller than the ones rescued by hCtr1, pointing to toxicity caused by copper overload. In contrast to the complementation ability of hCtr1 and *Drosophila* Ctr1 transgenes, we could not rescue the *Ctr1A*²⁵ mutants by raising them on food containing different concentrations of copper, even when the parents were also kept on food supplemented with copper (Fig. 3b). In an earlier study, a limited number of *Ctr1A*²⁵ mutants could be rescued by copper supplementation [21].

Discussion

To our surprise we found that hCtr1, despite its limited ability to function as a copper importer in *Drosophila*, rescued the *Ctr1A*²⁵ mutation at least as well as a transgene of *Drosophila* Ctr1A. We also showed that *Drosophila* Ctr1B and Ctr1C transgenes were able to rescue the mutant (although only transgenes with milder overexpression effects than observed with the Ctr1A transgene used in the complementation assay). These findings show a fundamental conservation in function between the mammalian and *Drosophila* Ctr1 proteins. Given the evidence of overexpression toxicity when the rescue was attempted with the *Drosophila* proteins, the comparatively lower copper-import efficiency of the human protein in the *Drosophila* environment could in fact have been beneficial in the rescue experiment. It is, however, puzzling that the lethality of *Ctr1A*²⁵ null mutants could not (Fig. 3b) or could only poorly [21] be rescued by copper supplementation. Although this might mean that the presence of Ctr1 importers is required for survival at least in some tissues, even though copper uptake does not have to be as efficient as with the endogenous Ctr1A gene, it also raises the

possibility that—in addition to the copper-uptake function—there is a copper-independent function in development shared by Ctr1-type copper transporters. Recently, overexpression of Ctr1 was reported to interfere with FGF signaling during early embryogenesis of *Xenopus laevis*, apparently independent of its role in copper transport [24]. Thus, it will be interesting to see whether hCtr1 transgenes which are defective in copper transport would still complement the *Ctr1A*²⁵ mutation.

Recent studies have revealed that the Ctr1 importers have diverged in their function. Whereas Ctr1A is the “housekeeping” copper importer with a ubiquitous expression pattern [21], Ctr1B helps to adjust copper uptake in the intestine, responding to copper demand and providing protection from excess copper in the food [14, 15, 22]. The similar behavior of Ctr1A and Ctr1B both in the overexpression (copper toxicity) assays and in the complementation experiment suggests that the functional differences between Ctr1A and Ctr1B are mainly to be explained by the differences in their spatial and temporal expression pattern, i.e., by the control of their transcription through differing *cis*-regulatory elements, but not by fundamental differences in the molecular function of the proteins.

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